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Importance of the *nef* Gene for Maintenance of High Virus Loads and for Development of AIDS

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Summary

When rhesus monkeys were infected with a form of cloned SIVmac239 having a premature stop signal at the 93rd codon of *nef*, revertants with a coding codon at this position quickly and universally came to predominate in the infected animals. This suggests that there are strong selective forces for open functional forms of *nef* in vivo. Although deletion of *nef* sequences had no detectable effect on virus replication in cultured cells, deletion of *nef* sequences dramatically altered the properties of virus in infected rhesus monkeys. Our results indicate that *nef* is required for maintaining high virus loads during the course of persistent infection in vivo and for full pathologic potential. Thus, *nef* should become a target for antiviral drug development. Furthermore, the properties of virus with a deletion in *nef* suggest a means for making live-attenuated strains of virus for experimental vaccine testing.

Introduction

Human immunodeficiency virus types 1 and 2 (HIV-1, HIV-2) and related lentiviruses of other species differ quite dramatically from other nonlentiretroviruses in the number of genes that they carry (for review see Cullen and Greene, 1990). All replication-competent retrovirus genomes contain *gag* (group-specific core antigen), *pol* (polymerase), and *env* (envelope) genes. HIV-1 has at least six additional genes called *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef*; HIV-2 and simian immunodeficiency virus from macaque (SIVmac) contain a similar set of accessory genes, *vif*, *vpr*, *vpx*, *tat*, *rev*, and *nef*. The *vif*, *vpr*, *vpx*, *vpu*, and *nef* genes have been termed "nonessential" since they can be deleted without completely abrogating the ability of virus to replicate (Fisher et al., 1987; Strebel et al., 1987, 1988; Ogawa et al., 1989; Cohen et al., 1988, 1990; Guyader et al., 1989; Luciw et al., 1987; Terwilliger et al., 1986). The conservation of these nonessential genes in distinct lentiviruses of different primate species argues for an important role in the virus life cycle.

The similarities between SIV and HIV in genomic organization, genetic sequence, and biologic properties suggest that SIV systems provide valid models for the study of acquired immunodeficiency syndrome (AIDS) pathogenesis (for reviews see Desrosiers, 1990; Desrosiers and Ringler, 1989). SIV closely parallels HIV in the use of the CD4 molecule as receptor (Daniel et al., 1985; Kannagi et al., 1985), in the regulation of viral expression (Malim et al., 1989; Sakai et al., 1990; Viglianti et al., 1990), and in the ability to cause AIDS (Daniel et al., 1985; Letvin et al., 1985; Baskin et al., 1988). Features of the AIDS-like disease caused by SIVmac in rhesus monkeys include CD4 depletion, opportunistic infections, lymphoid depletion, emaciation, and encephalitis, all characteristic of HIV-1-induced disease in humans. Molecularly cloned SIVmac239 causes AIDS and death in approximately 40% of rhesus monkeys within 6 months of inoculation (Kestler et al., 1990); the remaining 60% develop a more protracted disease course. The complete genetic sequence of the SIVmac239 infectious clone has been determined (Regier and Desrosiers, 1990). We used this cloned virus to study the importance of the *nef* gene for virus replication and for the development of AIDS.

While it is clear that the HIV-1 *nef* gene can be deleted without abrogating the ability of virus to replicate (Luciw et al., 1987; Terwilliger et al., 1986; Fisher et al., 1986; Ratner et al., 1985), conflicting reports have appeared regarding a possible negative influence of this gene product on the rate or extent of virus replication (Terwilliger et al., 1986; Luciw et al., 1987; Niederman et al., 1989; Kim et al., 1989; Hammes et al., 1989). In fact, the term "*nef*" is an acronym for "negative factor." Early reports (Terwilliger et al., 1986; Luciw et al., 1987) showed a small negative influence of the *nef* gene on virus replication. Subsequent studies reported even more dramatic down-regulatory effects of *nef* (Ahmad and Venkatesan, 1988; Niederman et al., 1989; Cheng-Mayer et al., 1989). However, Kim et al. (1989) and Hammes et al. (1989) found no effect of *nef* on HIV-1 replication nor on HIV-1 long terminal repeat (LTR)-driven CAT expression.

In the studies reported here, SIVmac239 replication in vitro did not differ detectably whether *nef* was present or deleted. However, dramatic differences were observed in the properties of cloned SIVmac239 variants differing only in their *nef* genes during the course of infection of rhesus monkeys. Our data indicate that *nef* is required for maintaining high virus loads during the course of persistent infection in vivo and that *nef* is required for full pathogenic potential.

Results

Cloned Virus Construction

The infectious SIVmac239 clone as isolated has a premature in-frame TAA stop signal at the 93rd codon of *nef* (Regier and Desrosiers, 1990). The TAA (stop) was changed to GAA (Glu) by oligonucleotide-directed site-specific mutagenesis with an M13 template to create SIVmac239/*nef*-open. GAA (Glu) is the codon used at this position in other infectious molecular clones of SIVmac

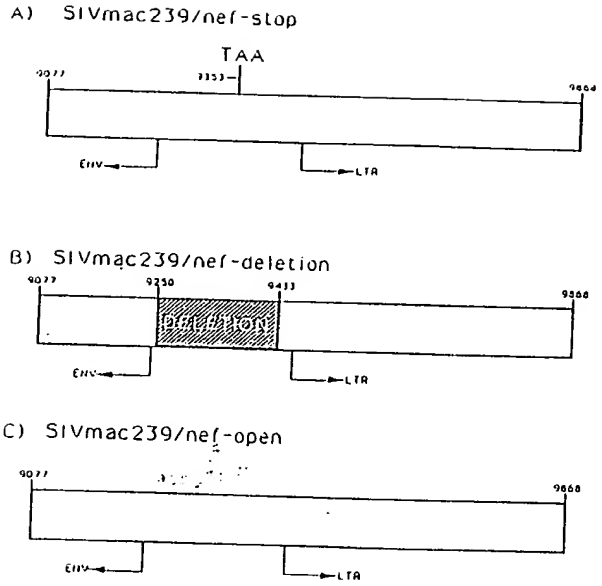


Figure 1. Schematic Representation of *nef* Genes Present in Infectious Molecular Clones

The *nef* reading frame of SIVmac239 begins at nucleotide 9077 and terminates at 9868 (numbering of Regier and Desrosiers, 1990). The amino-terminal portion of the *nef* reading frame overlaps the 3′ end of the envelope (*env*) gene. The carboxy-terminal portion of the *nef* reading frame overlaps the 3′ LTR. The *nef* protein is encoded by the +1 reading frame relative to *env*. The *env* open reading frame terminates at position 9243 and the 3′ LTR begins at nucleotide 9462.

(A) SIVmac239/nef-stop contains a stop signal (TAA) at position 9353–9355, the 93rd codon of the gene.

(B) SIVmac239/nef-deletion was derived from SIVmac239/nef-stop by oligonucleotide-directed site-specific deletion mutagenesis. Nucleotides 9251 to 9432 are deleted in SIVmac239/nef-deletion.

(C) SIVmac239/nef-open was also constructed by oligonucleotide-directed site-specific mutagenesis. The thymidine at position 9353 was changed to a guanosine, changing TAA (stop) to GAA (Glu).

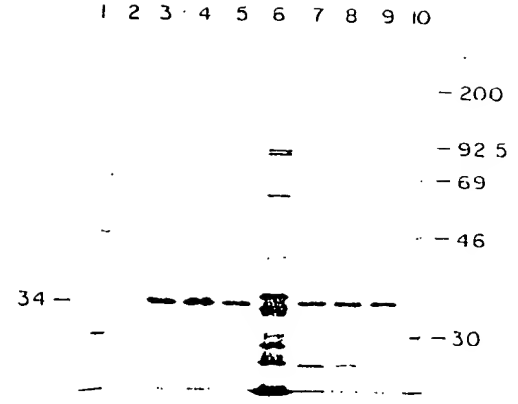


Figure 2. Immunoprecipitation of Vaccinia-Encoded *nef* Protein and SDS Gel Analysis

^{35}S-labeled cell lysates from vaccinia *nef*–infected cells were reacted with various sera. Lanes 2–9 are immunoprecipitations of vaccinia-encoded *nef* ^{35}S-labeled lysates with the following sera: lane 2, Mm206-86 preinoculation uninfected control sera; lane 3, Mm322-86 sera taken 10 weeks after SIV (*nef*-stop) infection; lane 4, Mm322-86 sera taken 26 weeks after SIV (*nef*-stop) infection; lane 5, Mm243-86 sera taken 13 weeks after SIV (*nef*-stop) infection; lane 6, mouse sera following infection with vaccinia-encoded *nef* virus; lane 7, sera from Mm44-84, a captive rhesus monkey found infected with SIV; lane 8, Mm243-86 sera taken 76 weeks after SIV (*nef*-stop) infection; lane 9, Mm243-86 sera taken 54 weeks after SIV (*nef*-stop) infection. Lanes 1 and 10 contain radioactive molecular weight markers having the indicated molecular weights in thousands. Molecular weight markers are ^{14}C methylated proteins from Amersham: myosin molecular weight, 200,000; phosphorylase-b, 92,500; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; lysozyme, 14,300.

(Chakrabarti et al., 1987; H. Kestler and R. Desrosiers, unpublished data). Oligonucleotide-directed site-specific mutagenesis was also used to create a 182 bp deletion in *nef*. This deletion was carefully constructed so as not to affect overlapping *env* sequences on the left or LTR sequences on the right (Figure 1). *nef* sequences are in a shifted reading frame 3′ to this deletion. These three forms of cloned virus (SIVmac239/nef-stop, SIVmac239/nef-open, SIVmac239/nef-deletion) differing in their *nef* genes are otherwise completely isogenic.

A vaccinia virus recombinant with nef-open sequences from SIVmac239 was used for expression studies. This vaccinia recombinant expressed a protein of a size expected for SIVmac *nef* (34 kd) that could be specifically immunoprecipitated by SIV$^+$ rhesus monkey sera (Figure 2). Additional experiments demonstrated that the SIVmac239 *nef* was myristylated (V. Stallard, G. Mazzara, D. Panicali, and H. Kestler, unpublished data), as is HIV-1 *nef* (Guy et al., 1987). The apparent molecular weight of the SIVmac239 *nef* product on SDS gels (34 kd) is somewhat larger than that reported previously for HIV-1 *nef* (25–29 kd) (Allan et al., 1985; Kaminchik et al., 1990, 1991; Zweig et al., 1990), consistent with the SIVmac239 *nef* reading frame being 53 amino acids longer than the HIV-1 *nef* reading frame.

Replication in Cultured Cells

The effect of *nef* mutations on replication of SIVmac239 was assessed in the human cell line CEMx174, in primary cultures of rhesus monkey peripheral blood lymphocytes (PBLs), and in primary rhesus monkey alveolar macrophage cultures.

DNA was transfected into CEMx174 cells, and aliquots were taken at periodic intervals for quantification of virus in the cell-free supernatant (Figure 3A). No significant differences in the replication of the three cloned viruses differing only in their *nef* genes were observed. When virus stocks were used to infect CEMx174, we similarly observed no significant differences in the rate or extent of virus replication in CEMx174 cells (data not shown). These conclusions are based on four independent experiments designed to examine the effect of *nef* on virus replication in CEMx174 cells.

The SIVmac142 infectious molecular clone contains an open *nef* gene (Chakrabarti et al., 1987). A frameshift was created at the 70th codon of the *nef* gene in SIVmac142 by filling in the protruding ends produced by cleavage with NcoI. Introduction of this frameshift mutation into the *nef* gene of SIVmac142 did not detectably alter the ability of virus to replicate in HUT-78 cells (data not shown).

Cloned SIVmac239 DNAs were also transfected into primary rhesus monkey PBLs that had been stimulated with phytohemagglutinin and were growing in the presence of interleukin-2. Following transfection of initial cultures, virus production was quite low in repeated experiments, probably due to the transient nature of these primary lymphocyte cultures (Figure 3B). However, strong virus replication was observed when supernatants from the initial transfected cells were used to infect fresh rhesus monkey PBL cultures (Figure 3C). Significant differences in the replication of the three cloned viruses differing only in their *nef* genes were not observed (Figure 3C). When controlled doses of virus were used to infect rhesus monkey PBL cultures at a low multiplicity of infection, we similarly observed no significant differences in the rate or extent of virus replication (Figure 3D). Four independent experiments failed to show any significant effect of *nef* on virus replication in rhesus monkey PBLs.

Since the SIVmac239 cloned virus replicates poorly in primary rhesus monkey alveolar macrophage cultures regardless of the status of the *nef* gene, we were not able to examine directly the effects of *nef* on replication of SIVmac239 in these macrophage cultures. However, K. Mori et al. (unpublished data) have recently isolated a cloned variant of SIVmac239 with eight amino acid changes in the envelope gene that replicates approximately three orders of magnitude better in alveolar macrophages than the parental SIVmac239. We thus used this derivative of SIVmac239, called SIVmac239/316Em, to examine the effect of *nef* on virus replication in primary rhesus monkey alveolar macrophages. A restriction fragment containing the envelope gene of SIVmac239/316Em was exchanged with SIVmac239 *env* in the three molecular clones that differ in their *nef* genes. SIVmac239/316Em virus stocks, with nef-open, nef-stop, and nef-deletion, were used to infect CEMx174 cells and primary rhesus monkey alveolar macrophage cultures. Virus containing 32 ng of p27 antigen was used for each infection. No significant differences in virus replication were observed (Figures 3E and 3F).

Reversion of Nef-Stop in Rhesus Monkeys

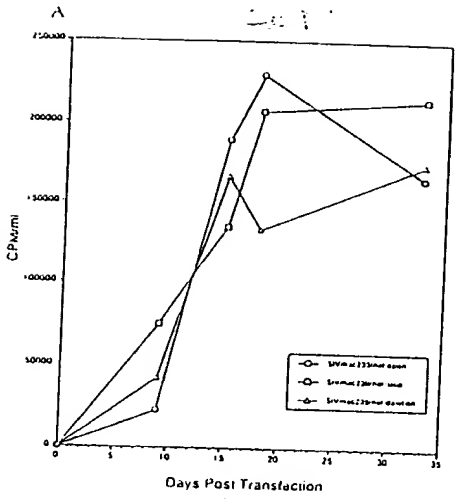
We proceeded to infect rhesus monkeys with the three SIVmac239 clones differing only in their *nef* genes. Five rhesus monkeys were inoculated with SIVmac239/nef-stop, seven with SIVmac239/nef-open, and six with SIVmac239/nef-deletion. All animals became infected since we were able to recover SIV repeatedly from their peripheral blood (Figure 4).

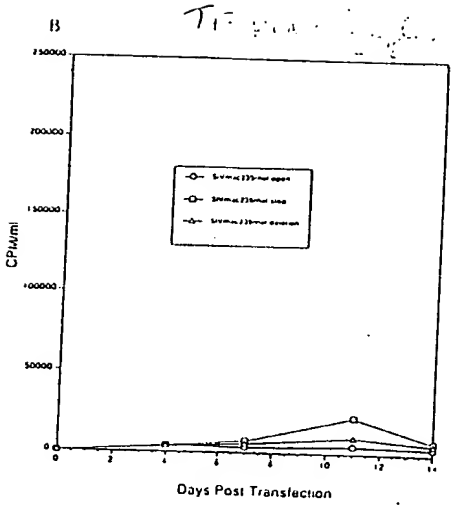
The status of the *nef* gene in rhesus monkeys that had been infected with SIVmac239/nef-stop was examined. DNA spanning the *nef* gene was amplified and cloned from cells infected with SIV recovered from two different rhesus monkeys infected with SIVmac239/nef-stop. Clones were obtained from one rhesus monkey (Macaca mulatta [Mm] 316-85) at the time of death with AIDS (21 weeks) and from another rhesus monkey (Mm243-86) at 6, 69, and 93 weeks after inoculation during the asymptomatic stage of persistent infection. In eight of eight clones examined, the stop signal at the 93rd codon had reverted to a coding codon (Figure 5). Four different types of reversion of the TAA stop codon were observed in these two animals: CAA (Gln), GAA (Glu), AAA (Phe), and TAC (Tyr). More recently, we have analyzed clones of SIV recovered just 2 weeks after infection of another rhesus monkey, Mm326-87. Even at this very early time point during the course of infection, four of four clones that were analyzed had mutations at the 93rd codon that reopened the *nef* reading frame (Figure 5, clones 9–12). The mutations observed were TAT (Tyr), CAA (Gln), GAA (Glu), and a 9 bp deletion that removed the stop codon (Figure 5). Fourteen of 15 DNA clones obtained by the polymerase chain reaction directly from brain and lymph node tissue of Mm316-85 also displayed reversion to a coding codon at the 93rd position of *nef* (T. Kodama and R. Desrosiers, unpublished data).

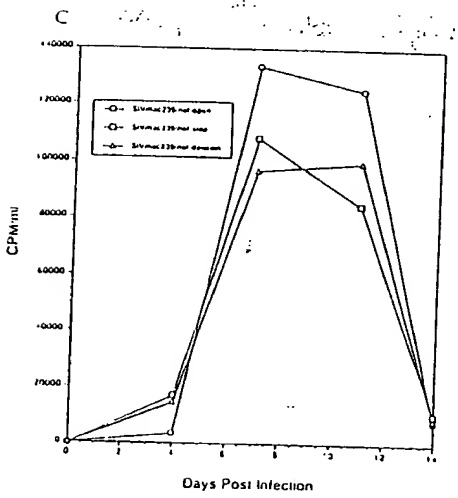
Virus Load in Rhesus Monkeys

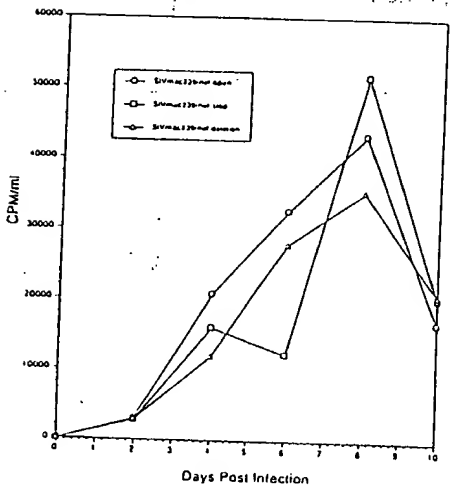
While all rhesus monkeys became infected with the different variants of SIVmac239, virus recovery from animals that received SIVmac239/nef-deletion became increasingly difficult with time (Figure 4). We thus performed experiments to estimate virus burden in rhesus monkeys infected with the three forms of SIVmac239. By serially diluting (3-fold) peripheral blood mononuclear cells (PBMCs) starting at 10^6 cells, the number of PBMCs needed to recover SIV by co-cultivation was quantitated (Ho et al., 1989). When measured at various times from 14 to 76 weeks after infection, the virus burden in rhesus monkeys that received SIVmac239/nef-deletion was consistently at least 100-fold lower than those in rhesus monkeys that received SIVmac239/nef-open and SIVmac239/nef-stop (Table 1). It is perhaps not surprising that the virus burdens were similar in rhesus monkeys that received SIVmac239/nef-stop vs. SIVmac239/nef-open since the stop codon in *nef* reverts to a coding codon in vivo (see above). The range of virus loads in this assay varied from approximately 1 in 1000 to 1 in 20,000 PBMCs in the SIVmac239/nef-open and SIVmac239/nef-stop groups (Table 1). This is a range very similar to what has been reported previously for HIV-1-infected people (Ho et al., 1989). Although SIV was not recovered from SIVmac239/nef-deletion-infected rhesus monkeys using 10^6 PBMCs, virus was occasionally recovered using greater than 10^6 PBMCs even longer than 1 year after infection.

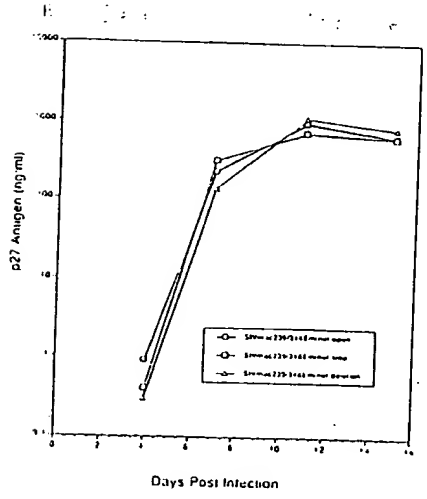
Inguinal lymph node biopsies were taken from six of the experimentally infected rhesus monkeys at 12 months after infection and examined histologically for morphologic alterations and immunohistochemically for the presence of SIV *gag* antigen. Three of these animals received SIVmac239/nef-stop (Mm124-79, Mm54-83, Mm326-87) and one received SIVmac239/nef-open (Mm206-86). All four of these animals had lymph nodes in various stages of follicular hyperplasia (Figure 6A), a pattern associated with

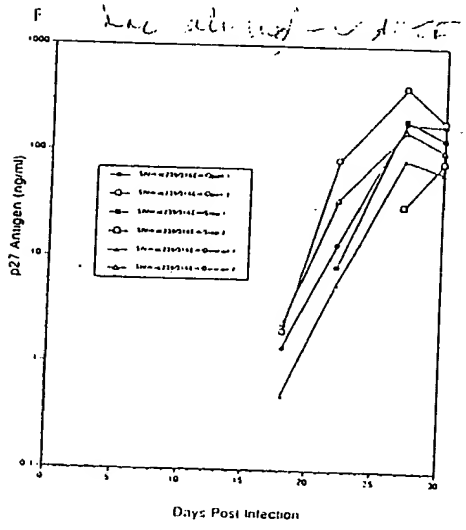

A
CPM/ml
Days Post Transfection


B
CPM/ml
Days Post Transfection


C
CPM/ml
Days Post Infection


D
CPM/ml
Days Post Infection


E
p27 Antigen (ng/ml)
Days Post Infection


F
p27 Antigen (ng/ml)
Days Post Infection

	NEF-STOP					NEF-OPEN							NEF-DELETION					
wk 2	+	+	+	+	+	+	+	+	—	+	+	+	+	+	+	—	+	+
wk 4	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
wk 6	+	ND	ND	+	+	ND	ND	+	+	+	ND	ND	ND	ND	+	+	ND	ND
wk 8	ND	+	ND	+	+	+	+	+	+	+	+	+	+	—	—	+	—	+
wk10	+	+	+	+	+	+	+	+	+	+	ND	+	—	+	+	—	ND	ND
wk12	ND	ND	ND	—	—	ND	ND	—	+	+	+	ND	ND	ND	—	+	—	+
wk14	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	—	ND	ND	ND	ND
wk16	ND	+	+	+	+	+	+	ND	ND	ND	+	ND	+	+	ND	ND	—	—
wk18	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	—	ND	ND	ND	ND
wk20	ND	+	ND	+	+	+	+	+	+	+	+	ND	—	—	+	+	—	+

Figure 4. SIV Recovery from Inoculated Animals

Blood samples from animals that received SIVmac239/nef-open, SIVmac239/nef-stop, and SIVmac239/nef-deletion were used for SIV recovery. Plus signs, positive; minus signs, negative; ND, not tested.

gross lymphadenopathy and early SIV and HIV-1 disease (Chalifoux et al., 1987; Armstrong and Horne, 1984). The lymph nodes from these four all contained SIV antigen within enlarged germinal centers in a dendritic staining pattern (Figure 6B) that previously has been shown to be associated with follicular dendritic cells (Ringler et al., 1989). In contrast, lymph nodes from the two animals that received SIVmac239/nef-deletion (Mm118-87 and Mm71-88) did not contain hyperplastic follicles (Figure 6C) and SIV *gag* protein was not detected (Figure 6D).

To gauge the extent of virus replication during the initial weeks following infection, p27 *gag* antigenemia was quantitated in cell-free plasma of all animals with an antigen capture assay. At 2 weeks after infection, significant levels of p27 protein were detected in seven of seven animals that received SIVmac239/nef-open and in five of five animals that received SIVmac239/nef-stop (Table 2). Levels of p27 protein ranged from 0.1 to 6.7 ng/ml. However, p27 protein was below the level of detection (approximately 0.05 ng/ml) in all six rhesus monkeys that received SIVmac239/nef-deletion at all times tested, weeks 2, 4, and 6 after inoculation (Table 2).

Antibody Response

Antibody responses were also monitored in these experimentally infected animals. Two of the rhesus monkeys that received SIVmac239/nef-open (Mm132-87 and Mm221-88) and two that received SIVmac239/nef-stop (Mm316-85 and Mm452-87) had only weak antibody responses, and these four died with AIDS within 7 months of infection (see below). The correlation of weak antibody response with rapid death with AIDS has been observed repeatedly in the past (Daniel et al., 1987; Kestler et al., 1990). The weak antibody responses of two of these animals are shown in Figure 7A. Among the strong antibody responders, there was no significant difference in the strength of the antibody response that could be correlated with the status of the *nef* gene (Figures 7A and 7B). Thus, despite a much lower

Figure 3. Effect of *nef* on Virus Replication

(A) Transfection of the lymphoid cell line CEMx174 with the three molecular clones differing only in *nef*. Virus production was monitored by assay of transcriptase activity (cpm) in the cell-free supernatant. The symbols used to identify the three viruses are as follows: circles, SIVmac239/nef-open; squares, SIVmac239/nef-stop; triangles, SIVmac239/nef-deletion.

(B) Virus production following transfection of macaque lymphocytes with the three molecular clones. Symbols are the same as in (A).

(C) Virus-containing supernatants from 11 day transfected lymphocyte cultures were used to infect fresh macaque lymphocytes (day 0). Virus production was measured by reverse transcriptase activity. Symbols are the same as in (A).

(D) Replication of SIVmac239/nef-open, SIVmac239/nef-stop, and SIVmac239/nef-deletion in macaque lymphocytes using a normalized low multiplicity of infection. Frozen virus containing 5000 cpm of reverse transcriptase activity prior to freezing from day 7 in (C) was used to infect freshly stimulated macaque lymphocytes. Assay of reverse transcriptase activity was performed on cell-free supernatant every 2 days following infection. Symbols used are the same as in (A).

(E) Virus stocks were prepared 11 days after transfection of CEMx174 cells with SIVmac239/316Em/nef-open (circles), SIVmac239/316Em/nef-stop (squares), and SIVmac239/316Em/nef-deletion (triangles). Virus containing 32 ng of p27 core protein was used to infect CEMx174 cells, and virus production was monitored by assay of p27 protein in the cell-free supernatant by antigen capture.

(F) Virus containing 32 ng of p27 core protein as in (E) was used to infect primary rhesus monkey alveolar macrophages from a single animal in wells of a 24-well tray. Each sample was tested in duplicate. Supernatant samples were removed at the times indicated for assay of p27 core protein by antigen capture. Duplicates are indicated by open and closed symbols.

CLONE	ANIMAL NUMBER	CODON	WEEKS POST INOCULATION	MUTATION
1	Mm243-86	93	93	TAA (STOP) → GAA (GLU)
2	Mm243-86	93	93	TAA (STOP) → AAA (LYS)
3	Mm243-86	93	69	TAA (STOP) → GAA (GLU)
4	Mm243-86	93	6	TAA (STOP) → GAA (GLU)
5	Mm243-86	93	6	TAA (STOP) → GAA (GLU)
6	Mm243-86	93	6	TAA (STOP) → GAA (GLU)
7	Mm316-85	93	21	TAA (STOP) → TAC (TYR)
8	Mm316-85	93	21	TAA (STOP) → CAA (GLN)
9	Mm326-87	93	2	TAA (STOP) → TAT (TYR)
10	Mm326-87	93	2	TAA (STOP) → CAA (GLN)
11	Mm326-87	93	2	TAA (STOP) → GAA (GLU)
12	Mm326-87	92-94	2	TAA (STOP) → DELETION OF STOP AND TWO FLANKING CODONS

Figure 5. Reversion of the Stop Codon in SIVmac239/nef-Stop In Vivo

Clones spanning the *nef* gene were obtained from animals infected with SIVmac239/nef-stop. The sequence at the 93rd codon of the *nef* gene is shown.

virus load, rhesus monkeys infected with SIVmac239/nef-deletion had strong antibody responses, similar to the responses of long-term survivors in the other groups. Antibody titers continued to increase or to persist at high levels for at least 12 months even in the rhesus monkeys that received SIVmac239/nef-deletion (Figure 7B). These results are consistent with persistent infection for at least 12 months.

Pathogenic Potential

Two of the five rhesus monkeys that received nef-stop virus and three of the seven that received nef-open virus have died so far, 2½ to 7 months after infection (Table 3). All five animals died with pathologic changes clearly consistent with SIV-induced AIDS. Pathologic findings included disseminated CMV infection, lymphoid depletion, thymic atrophy, emaciation, enterocolitis, a characteristic granulomatous encephalitis, and giant cell pneumonia. It is again not surprising that the nef-stop and nef-open forms of virus appear to have similar pathogenic potentials since nef-stop reverts to nef-open in vivo. All six rhesus monkeys that received SIVmac239/nef-deletion are alive to date and all six remain healthy. All six have been infected for over 12 months (median of 16 months). Furthermore, the CD4+ lymphocyte subset concentrations in these six rhesus monkeys that received SIVmac239/nef-deletion are normal (Table 1). In contrast, the rhesus monkeys infected with SIVmac239/nef-open and SIVmac239/nef-stop that

Table 1. Effects of *nef* on Virus Load and CD4 Lymphocyte Concentration

Animal #	Infecting SIVmac239	Weeks Postinfection	PBMCs Needed to Yield SIV[a]	% Lymphocytes as CD4	CD4 Lymphocyte Concentration[b]
292-88	nef-open	14	8,230		
358-88	nef-open	24	24,691		
358-88	nef-open	40	8,230		
358-88	nef-open	62	2,743	20.8	944
135-88	nef-open	40	24,691		
135-88	nef-open	62	8,230	25.5	1,354
155-88	nef-open	40	8,230		
155-88	nef-open	62	8,230	21.6	970
206-86	nef-open	44	914		
206-86	nef-open	76	24,691	13.9	531
128-89	nef-deletion	14	>1,000,000		
128-89	nef-deletion	42	>1,000,000	52.4	1,740
255-88	nef-deletion	14	>1,000,000		
255-88	nef-deletion	42	>1,000,000	43.1	1,232
397-88	nef-deletion	40	>1,000,000		
397-88	nef-deletion	62	>1,000,000	42.9	2,316
353-88	nef-deletion	40	>1,000,000		
353-88	nef-deletion	62	>1,000,000	41.3	3,209
118-87	nef-deletion	44	>1,000,000		
118-87	nef-deletion	76	>1,000,000	35.5	1,682
71-88	nef-deletion	44	>1,000,000		
71-88	nef-deletion	76	>1,000,000	37.2	2,648
124-79	nef-stop	44	8,230		
124-79	nef-stop	76	24,691	11.1	421
54-83	nef-stop	44	12,345		
54-83	nef-stop	76	12,345	12.6	NT[c]
326-87	nef-stop	44	2,743		
326-87	nef-stop	76	12,345	35.0	1,638

[a] Average of duplicate measurements. Serial 3-fold dilutions beginning at 10^4 were used.
[b] Number per mm^3.
[c] Not tested because of a lack of adequate sample.

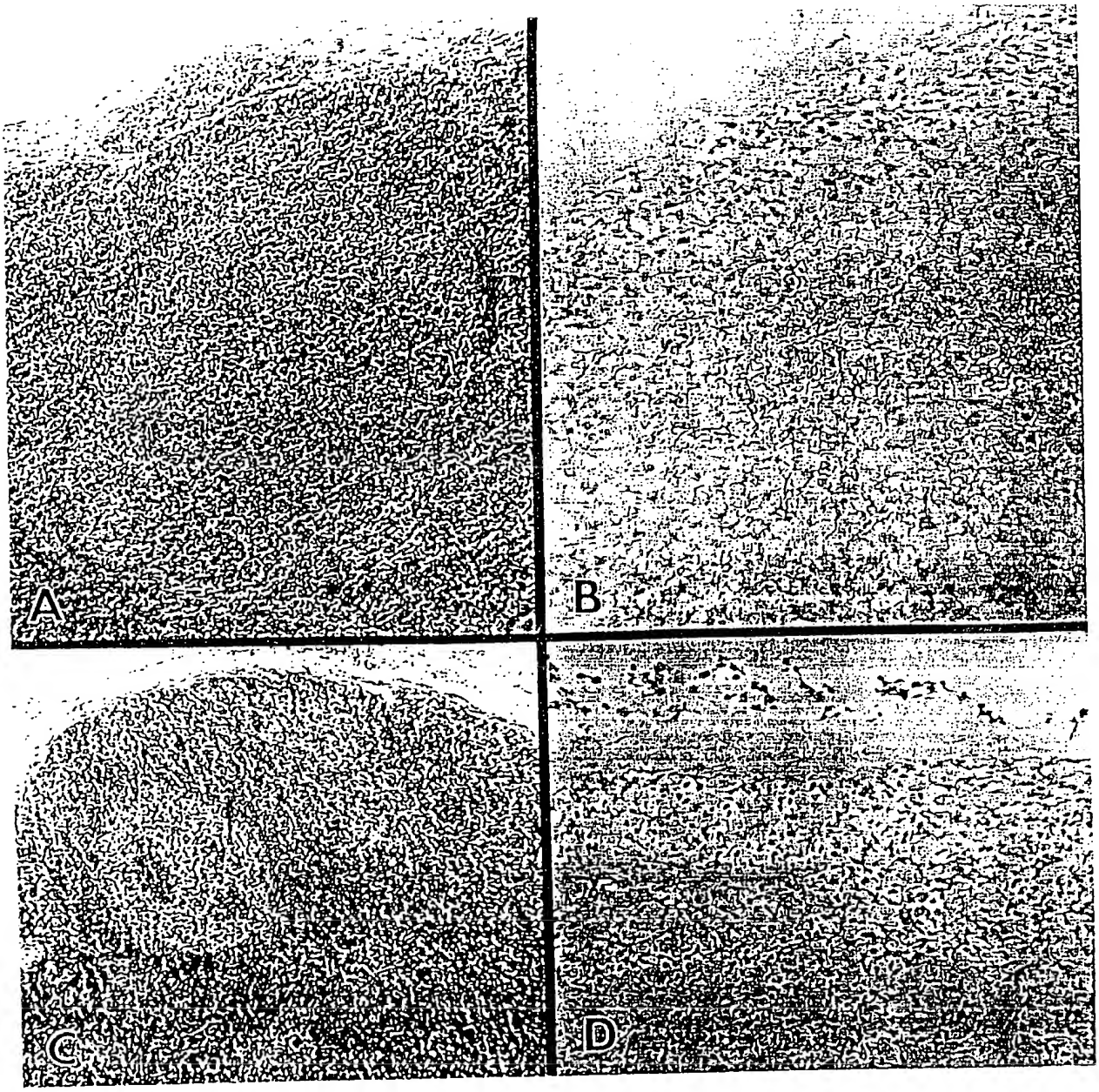


Figure 6. Inguinal Lymph Nodes from Animals Infected with Molecular Clones of SIVmac239

(A and B) Inguinal lymph node from Mm326-87 taken 12 months after infection with SIVmac239/nef-stop. There is profound follicular hyperplasia characterized by expansile germinal centers (A). SIV *gag* protein is localized in a dendritic pattern within these follicular centers (B). This staining pattern is typical of viral protein localization to resident follicular dendritic cells.

(C and D) Inguinal lymph node from Mm118-87 taken 12 months after infection with SIVmac239/nef-deletion. The nodal cortex contains follicles proportionally smaller than those in (A), and no SIV *gag* protein can be localized to the nodal parenchyma using immunohistochemical techniques. Magnifications are 90× (A and C), 360× (B), and 290× (D).

are still alive show declining $CD4^+$ lymphocyte subset concentrations (Table 1) and clinical signs of declining health. Examination of lymph node biopsies taken at 12 months also revealed clear histologic differences between animals that received nef-deletion virus (normal) and those that received nef-open or nef-stop (follicular hyperplasia) (see above).

Discussion

Similar to the reports of Kim et al. (1989) and Hammes et al. (1989) for HIV-1, we observed no significant influence of the *nef* gene on replication of SIVmac239 in cultured cells. Using standard procedures, virus replication was monitored in the human lymphoblastoid cell line CEMx174,

Table 2. Plasma Antigenemia

Infecting Virus	Animal #	ng/ml p27 Antigen[a]			
		Week 0	Week 2	Week 4	Week 6
nef-open	132-87	0.0	1.1	0.7	0.7
nef-open	206-86	0.0	0.1	0.0	0.0
nef-open	135-88	0.0	6.7	0.1	0.0
nef-open	358-88	0.0	0.5	0.0	0.0
nef-open	155-88	0.0	4.8	5.0	2.4
nef-open	292-88	0.0	0.7	0.1	NT[b]
nef-open	221-88	0.0	2.3	0.7	NT
nef-stop	316-85	0.0	5.5	1.0	3.8
nef-stop	326-87	0.0	2.0	0.0	0.0
nef-stop	452-87	0.0	1.8	0.5	1.9
nef-stop	54-83	0.0	0.1	0.0	0.0
nef-stop	124-79	0.0	0.1	0.0	0.0
nef-deletion	118-87	0.0	0.0	0.0	0.0
nef-deletion	71-88	0.0	0.0	0.0	0.0
nef-deletion	353-88	0.0	0.0	0.0	0.0
nef-deletion	397-88	0.0	0.0	0.0	0.0
nef-deletion	128-89	0.0	0.0	0.0	0.0
nef-deletion	255-88	0.0	0.0	0.0	0.0

[a] In plasma. The limit of detection is approximately 0.05 ng/ml. Week 0 is a preinfection sample taken immediately before inoculation of virus.
[b] Not tested (sample not available).

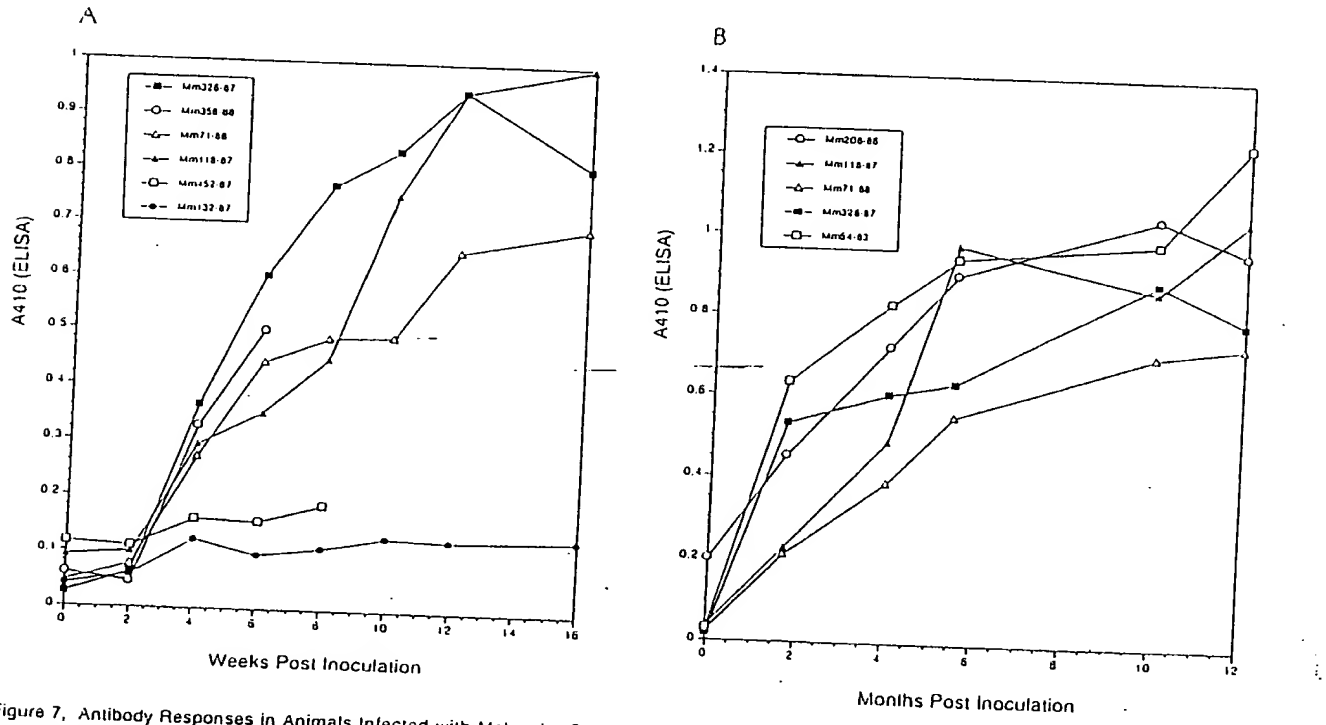


Figure 7. Antibody Responses in Animals Infected with Molecular Clones of SIVmac239

Antibody responses to SIV antigen by enzyme-linked immunosorbent assay (ELISA) are shown for eight animals infected with SIV molecular clones. (A) Antibody responses during the first 4 months following inoculation. Mm326-87 (closed squares) and Mm452-87 (open squares) received SIVmac239/nef-stop; Mm358-88 (open circles) and Mm132-87 (closed circles) received SIVmac239/nef-open; Mm118-87 (closed triangles) and Mm71-88 (open triangles) received SIVmac239/nef-deletion.

(B) Antibody responses during the first year after inoculation. Mm54-83 (open squares) and Mm326-87 (closed squares) received SIVmac239/nef-stop virus; Mm206-86 (open circles) received SIVmac239/nef-open virus; Mm118-87 (closed triangles) and Mm 71-88 (open triangles) received SIVmac239/nef-deletion.

Table 3. Pathogenic Potential of Molecularly Cloned Virus

Cloned Virus	Number Infected	Number of Deaths
SIVmac239/nef-stop	5	2
SIVmac239/nef-open	7	3
SIVmac239/nef-deletion	6	0

In the group that received SIVmac239/nef-stop, two (Mm316-85 and Mm452-87) died with AIDS 3½ and 5 months after infection, manifest by lentiviral encephalitis and granulomatous pneumonia or disseminated CMV infection. In the group that received SIVmac239/nef-open, all three animals (Mm132-87, 221-88, and 292-88) died with AIDS 2½, 7, and 6 months after infection, respectively. The AIDS-related sequelae found in these three animals included granulomatous pneumonia, disseminated CMV infection, emaciation, and thymic and lymphoid atrophy.

in primary rhesus monkey PBL cultures growing in interleukin-2, and in primary rhesus monkey alveolar macrophage cultures. The dispensability of *nef* for virus replication in these cell culture conditions is consistent with the frequent observation of defects in *nef* in infectious molecular clones of HIV-1, HIV-2, and SIVmac. Three of the four infectious molecular clones of SIVmac and HIV-2 that we have studied (Chakrabarti et al., 1987; Kestler et al., 1988; Naidu et al., 1988) have obvious defects in *nef*. We interpret this to mean that there is little or no selective pressure to maintain open functional forms of *nef* under standard cell culture conditions.

Conversely, there appears to be strong selective pressure to maintain open, functional forms of *nef* in infected rhesus monkeys. In animals infected with SIVmac239/nef-stop, the stop signal in *nef* quite quickly and universally reverted to a coding codon. Six different types of mutations were observed at the 93rd codon of *nef* in three animals infected with SIVmac239/nef-stop: GAA (Glu), TAC (Tyr), CAA (Gln), TAT (Tyr), AAA (Lys), and a 9 bp deletion. This specificity and diversity of mutations essentially rules out any sort of contamination or polymerase chain reaction artifact for these observations. The strong selective pressure to maintain open, functional forms of *nef* in infected animals indicates that *nef* is performing some critical function for the virus life cycle in vivo. Furthermore, these results suggest that the open form of *nef* in SIVmac239 is a functional one and can be used to study the role of *nef*. This latter point is important since there appears to be no selective pressure to maintain functional forms of *nef* in cell culture and no in vitro phenotype has been consistently associated with the elimination of *nef*.

Despite the absence of any clear effect of the 182 bp deletion within *nef* on virus replication in cultured cells, the properties of SIVmac239/nef-deletion in rhesus monkeys differed dramatically from counterpart nef-open and nef-stop viruses, which otherwise were completely isogenic. The major phenotypic effects associated with the deletion of *nef* sequences were maintenance of much lower virus loads and decreased pathogenic potential.

Decreased virus loads in animals that received SIVmac239/nef-deletion were observed by limiting dilution [illegible] of PBMCs from infected animals, by immunohistochemical staining of lymph node biopsies, and by measurement of plasma antigenemia during the initial weeks following infection. The "wild-type" virus loads of 1 in 1000 to 1 in 20,000 PBMCs are in the same range as has been reported previously for HIV-1-infected people (Ho et al., 1989). Virus loads in rhesus monkeys that received SIVmac239/nef-deletion were at least 100-fold lower. Nonetheless, rhesus monkeys that received SIVmac239/nef-deletion remained persistently infected for at least 12 months since we could occasionally recover SIV when more than 10^6 PBMCs were used for co-cultivation and infected animals maintained strong, stable antibody responses that persisted at high levels for at least 12 months. The reversion of nef-stop by 2 weeks in vivo explains why similar levels of plasma antigenemia were observed in nef-open and nef-stop groups during the initial weeks following infection.

Decreased pathogenic potential of SIVmac239/nef-deletion was evident from the lack of deaths for over 1 year in the group of six infected animals, their continued good health, the normal appearance of lymph node biopsies, and the maintenance of normal $CD4^+$ lymphocyte concentrations in all six animals. Among the 7 of 12 who are still alive following infection with SIVmac239/nef-open and SIVmac239/nef-stop, varying degrees of ill health are clearly evident. Clinical findings include decreasing $CD4^+$ lymphocyte subset concentrations, lymphadenopathy, anemia, diarrhea, and weight loss. None of these are evident in the six rhesus monkeys infected with SIVmac239/nef-deletion. More time and additional animals will be needed to demonstrate whether *nef* is an absolute requirement for the development of AIDS.

Although we have clearly demonstrated the importance of *nef* and have identified remarkable phenotypic differences associated with the deletion of *nef* in vivo, the functional role of *nef* remains unknown. One possibility is that the cell culture conditions used to measure virus replication do not accurately reflect virus production in the host. By this scenario, *nef* would play an important role in the replication of virus in the major cell types producing virus in vivo or under conditions present in vivo that are not reflected in cell culture. Another possibility is that *nef* may play some regulatory role in vivo in allowing virus to persist at high levels in the face of a strong host immune response. Such a role in persistence could be achieved, for example, by down-regulation of HLA class I antigens to avoid recognition by virus-specific cytotoxic T lymphocytes. Markedly decreased plasma antigenemia in rhesus monkeys that received SIVmac239/nef-deletion at 2 weeks after infection, before significant antibody levels can be detected in the peripheral blood, argues for the former possibility. Our current experiments are directed toward testing these hypotheses.

Our findings are not without important practical applications. Products of the so-called nonessential genes have by and large not been targeted for antiviral drug development because there has been no evidence that knocking them out would interrupt the disease process. Our results indicate that *nef* is a critical component of the virus' ability to induce AIDS. Thus, *nef* should become a target for anti-

viral drug development. In addition, our findings suggest a means for making live-attenuated vaccine strains for experimental testing.

Experimental Procedures

Plasmid Constructions

The SIVmac239 molecular clone has been subcloned into two segments, p239SpSp5′ and p239SpE3′, which yield infectious virus following ligation at a common SphI restriction site and transfection into susceptible cells (Kestler et al., 1990). All manipulations of the *nef* gene were performed with the 3′ subclone p239SpE3′. A 2206 bp SstI fragment of p239SpE3′ containing most of the *nef* gene from viral nucleotide 9230 to an SstI site in flanking cellular sequences was subcloned into M13mp18. This subclone, M13-239SstB, was used as a template for oligonucleotide-directed site-specific mutagenesis (Zoller and Smith, 1987). To create 239SpE3′/*nef*-open, an oligonucleotide having the sequence GTCATCATCTTCCTCATCTAT was synthesized. This oligonucleotide spans the stop signal at codon 93 of the *nef* gene in p239SpE3′. It is identical to the sequence of the antisense strand of SIVmac239 except the A at position 9353 has been changed to a C. This oligonucleotide was used as a primer to synthesize the minus strand of M13-239SstB and as a probe to screen for mutagenized clones. The SstI fragment containing an open *nef* gene was liberated from the M13 vector and reinserted back into the molecular clone p239SpE3′, creating p239SpE3′/*nef*-open.

A similar strategy was used to construct a 182 bp deletion in the *nef* gene. An oligonucleotide containing the sequence of the antisense strand nucleotides 9215–9250 and 9433–9469 (i.e., this 73-mer is missing 182 bp from 9251 to 9432) was synthesized to use as a primer for second strand synthesis on the M13-239SstB template. The SstI fragment containing the deletion was inserted back into p239SpE3′ creating p239SpE3′/*nef*-deletion. Viral DNA in the mutated M13 subclones was completely sequenced to verify the presence of the desired mutations and to confirm that no other changes occurred in viral DNA sequences.

The macrophage tropic clones SIVmac239/316Em/*nef*-open, SIVmac239/316Em/*nef*-stop, and SIVmac239/316Em/*nef*-deletion were constructed by replacing the SphI–SstI fragment 6450–9230 of the three right-half SIVmac239 molecular clones with the SphI–SstI fragment of SIVmac239/316Em.

Cells and Viruses

Human T cell lines HUT-78 and CEMx174 were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. Rhesus monkey PBMCs were purified by banding over a sodium diatrizoate Ficoll gradient (1077–1080 g/ml; Pharmacia). For rhesus monkey PBL cultures, PBMCs were treated with 1 μg/ml phytohemagglutinin for 48 hr, washed free of lectin, and maintained in RPMI 1640 containing 20% fetal calf serum, 10% interleukin-2 (Electronucleonics), 100 U/ml penicillin, and 100 μg/ml streptomycin. Virus recovery from animals was performed by purifying PBMCs as described above and co-cultivating with SIVmac-sensitive lymphoid cell lines CEMx174 or HUT-78. Cultures were maintained for 1 month and were assayed for the presence of reverse transcriptase (Naidu et al., 1988; Daniel et al., 1985) or were assayed for the presence of p27 SIV core protein by antigen capture (Coulter). A negative virus recovery indicates antigen capture or reverse transcriptase assay values not significantly over that of uninfected cells. The procedures for culture of primary rhesus monkey alveolar macrophages have been described (Ringler et al., 1989). Procedures for measurement of virus replication in cell lines, rhesus monkey PBL cultures, and rhesus monkey primary alveolar macrophage cultures have been described (Naidu et al., 1988; Desrosiers et al., submitted).

Radiolabeling of Cells and Immunoprecipitation of *nef*

A vaccinia recombinant expression vector containing *nef* gene sequences from 8965 to 9908 (PstI to RsaI) from p239SpE3′/*nef*-open was used to demonstrate *nef* protein expression. Owl monkey kidney cells infected with this vaccinia recombinant were labeled with 500 μCi of [^{35}S]methionine (1000 Ci/mmol; Amersham) for 20 hr in medium lacking methionine, washed twice with PBS, scraped from flasks, and centrifuged. Cells were frozen at −70°C and thawed in 1 ml of lysis buffer (0.5% NP-40, 0.15 M NaCl, 1 mM EDTA, 20 mM Tris–HCl [pH 7.5], 100 μg/ml PMSF; Desrosiers and Falk, 1981). Cells were incubated on ice for 20 min with vigorous vortexing every 3 min. The cell extract was centrifuged at 20,000 × g for 30 min at 4°C. Approximately 2 × 10^5 cpm from the supernatant fraction was diluted to 100 μl in lysis buffer, was preabsorbed to 100 μl of 20% protein A–Sepharose CL4B, and incubated on ice for 1 hr with gentle shaking. The nonspecific protein A–bound material was removed by centrifugation for 15 min at 13,600 × g at 4°C, and 5 μl of the indicated sera was added to each supernatant and incubated at 30°C for 30 min. Fresh 20% protein A–Sepharose CL4B (180 μl) was added, and each sample was incubated for 20 min at room temperature. The protein A–Sepharose CL4B complex was pelleted, washed seven times with lysis buffer, resuspended with 50 μl of SDS–PAGE sample buffer (2% SDS, 10% glycerol, 1.2 M β-mercaptoethanol, 60 mM Tris [pH 6.8], 0.001% bromphenol blue), and heated to 85°C for 10 min. Supernatant (15 μl) from the SDS-treated sample was loaded onto a 12% SDS–polyacrylamide gel (Laemmli, 1970). The gel was fixed for 1 hr in 5% methanol, 7.5% acetic acid, rinsed for 30 min in water, soaked for 1 hr in 1 M sodium salicylate, and dried. The dried gel was exposed to Kodak X-ray film for 3 days. Molecular weight markers are ^{14}C methylated proteins from Amersham: myosin molecular weight, 200,000; phosphorylase-b, 92,500; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; lysozyme, 14,300.

DNA Transfection

The 5′ and 3′ clones of SIVmac were cleaved with SphI and heated to 60°C for 10 min. Each right-half clone was ligated together with the left-half clone p239SpSp5′ using T4 DNA ligase. The ligated mixture was concentrated by ethanol precipitation. Six micrograms of the ligated DNA was used to transfect macaque lymphocytes or CEMx174 cells treated with DEAE–dextran (Sompayrac and Danna, 1981; Milman and Herzberg, 1981; Naidu et al., 1988).

Identification of Stop Codon Reversion

SIV was recovered from rhesus monkeys by co-cultivation of PBMCs with the SIV-sensitive line CEMx174 at the times indicated in Figure 5. DNA was extracted from infected CEMx174 cells according to the procedure of Hirt (1967). Oligonucleotide o239pcrnef7s having the sequence GTATACCGGATCCTCCAACCAATACTCCAG complementary to the sense strand 8982–9012 was synthesized along with o239pcrnef8as having the sequence CTTGATGTATAAATATCACTGCACTTCGC complementary to the antisense strand 9942–9970. Each oligonucleotide contained a single mismatched base: in o239pcrnef7s the A at base 8990 was changed to a G, creating a BamHI restriction endonuclease recognition site; o239pcrnef8as contains a C instead of an A at base 9965, yielding a PstI recognition site. These primers were used to amplify the *nef* gene from approximately 1 μg of Hirt supernatant DNA. The polymerase chain reaction was performed using Amplitaq (Cetus) under the conditions described by the supplier. The reactions were subjected to 30 thermal cycles of the following composition: 1 min at 94°C followed by 2 min at 55°C and 1 min at 72°C. After each cycle, 5 s were added to the 72°C segment. The amplified material was purified using a Sepharose CL4B (Pharmacia) spun column and self-ligated to high molecular weight. Concatemers were cleaved with PstI and BamHI. The cleaved DNA was cloned into the M13 bacteriophage vector M13mp18 at the BamHI and PstI sites. Plaques were screened for the presence of *nef* sequences, and the resulting *nef*-positive M13 phage DNA was sequenced by the dideoxy chain termination method of Sanger et al. (1977) using Sequenase (US Biochemical Corp.) as a polymerase.

Infection of Rhesus Monkeys and Antibody Response

The 18 rhesus monkeys used in this study were all infected by intramuscular inoculation of virus produced in rhesus monkey PBL cultures from ligated plasmid subclones. Mm243-86, used for the data presented in Figure 5, was infected by virus produced in HUT-78 cells following transfection with the parental SIVmac239 λ phage (Burns and Desrosiers, 1991). All experimental animals were kept in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on the Care and Use of Laboratory Animals, National Research Council. Heparinized

blood samples were obtained at periodic intervals following infection. Plasma was separated from cells by centrifugation and stored frozen at −70°C. Thawed plasma was used at a straight 1:20 dilution (Figure 7A) or 1:100 dilution (Figure 7B) for reactivity to purified SIVmac by ELISA (Daniel et al., 1988). CD4 lymphocyte concentrations were determined commercially at TSI Mason Research Laboratories (Worcester, MA) by Dr. Michael Wyand. Immunohistochemical localization of SIV *gag* protein was performed as previously described (Ringler et al., 1989).

Virus Load Determinations

PBMCs were obtained from heparinized blood samples by banding over a sodium diatrizoate Ficoll gradient as described above. The concentration of PBMCs was measured with a hemocytometer, and serial 3-fold dilutions starting with 1.0×10^6 cells were performed in duplicate. The diluted PBMCs were co-cultivated with 1×10^5 CEMx174 cells in a volume of 1 ml. On day 3, 1 ml of RPMI 1640 containing 10% fetal calf serum was added to each dilution, and thereafter cultures were split 1:2 two times per week until day 21 when the cultures were assayed for virus production by reverse transcriptase (Naidu et al., 1988) or SIV antigen capture (Coulter).

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